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Riboflavin Production during Growth of *Micrococcus luteus* on Pyridine†

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***Micrococcus luteus* produced 29 μM riboflavin during growth on 6.5 mM pyridine but not during growth on other substrates. On the basis of the results of radiolabelling studies, riboflavin was not directly synthesized from pyridine. Pyridine may interfere with riboflavin biosynthesis or elicit a general stress response in *M. luteus*.**

Numerous bacteria capable of degrading pyridine have been isolated from various environments, especially soil. As yet, none of these organisms has produced detectable quantities of metabolites prior to ring fission (6). When grown on pyridine as a carbon, nitrogen, and energy source, *Micrococcus luteus* produces a yellow fluorescent pigment of unknown identity (7). We have conducted experiments to identify the chemical nature of this yellow product and to determine if it represents an intermediate in pyridine degradation. Pigment was not detected during growth of the organism on any substrate (glucose, 4-aminobutyric acid, succinate, or nutrient broth) other than pyridine, except when pyridine was added as a second substrate. Most of the pigment appeared during the late exponential and stationary phases of growth in batch cultures, and its production ceased (as did pyridine degradation) when oxygen was withheld from the culture. The optimum concentration of pyridine for both growth of the organism and pyridine degradation was 13 mM. Above 25 mM, pyridine temporarily inhibited growth, pyridine degradation, oxygen uptake, and pigment production.

M. luteus (ATCC 49442) was grown in a defined medium (7) containing pyridine (13 mM), mineral salts, and thiamin (2 μM). Cells were removed from the culture by centrifugation when the concentration of pigment reached a maximum. The pigment was recovered by extracting lyophilized culture medium with hot (50°C) methanol. Salts were removed by differential crystallization in a rotary evaporator. The resulting yellow fine crystalline material contained three fluorophores, as determined by thin-layer chromatography (TLC) on silica gel plates (acetonitrile-isopropanol-water, 7.5:2.1:1.1). The dominant fluorophore was further purified by solid-phase extraction and preparative TLC (conditions described above).

On the basis of chromatographic retention in reverse-phase high-pressure liquid chromatography (HPLC) and on normal-phase TLC plates, the three fluorophores were tentatively identified as riboflavin, lumiflavin, and lumichrome and thus deemed unlikely candidates for degradative intermediates or products of pyridine polymerization. Lumiflavin and lumichrome were also formed from riboflavin in uninoculated medium, suggesting that they were produced abiotically. Lumiflavin and lumichrome have been reported as the primary abiotic degradation products for riboflavin in aqueous solution (3).

Identity of the dominant fluorophore as riboflavin was confirmed by several spectroscopic techniques. Analysis by X-ray diffraction (CuKα, 35 kV) of freshly crystallized pigment revealed a complex crystalline structure with numerous interplanar d-spacings. At least 11 strong peaks (I/I_0 = 15% or more) were observed. These peaks (1.02, 0.84, 0.76, 0.50, 0.45, 0.40, 0.36, 0.35, 0.33, 0.32, and 0.30 nm) matched closely with reported values for riboflavin A and exactly matched those for an authentic sample of riboflavin prepared in the same manner as the pigment sample. The Fourier transform infrared spectrum (KBr pellet) of the pigment agreed with that of authentic riboflavin (Fig. 1), as did fluorescence excitation (λ_{max} at 267 and 365 nm) and emission (λ_{max} = 520 nm) spectra, as well as the UV-visible absorption spectrum (λ_{max} = 443 nm).

Further evidence for the identity of the pigment was taken from bioactivity experiments. Bioactivity of the isolated pigment produced by *M. luteus* was determined with bioassays (8) using *Lactobacillus casei* (ATCC 7469) grown in liquid riboflavin assay medium (Difco Laboratories, Detroit, Mich.) containing 0 to 0.15 μM riboflavin. The quantitative growth responses of *L. casei* to the isolated pigment or authentic riboflavin supplied at a similar concentration did not differ significantly, suggesting that the pigment was bioactive. It should be noted that *L. casei* may respond to some biologically inactive analogs of riboflavin, including diethyl, methyl, and deoxy analogs (8).

Riboflavin did not arise directly from pyridine, as demonstrated by radiotracer experiments. In these experiments, *M. luteus* was grown in 10-ml cultures containing 6.5 mM 2,6-^{14}C-labeled pyridine (3.1 Ci mol^{-1} or 0.02 μCi ml^{-1}) in 160-ml serum bottles sealed with rubber septa. Radiochromatograms were prepared simultaneously with UV chromatograms, using reverse-phase HPLC. Several UV-absorbing substances eluted during the first 10 min of chromatograms of samples taken at 24, 48, or 72 h of incubation. Only one of the peaks, which eluted with the solvent front, was radioactive and was probably a polar metabolite (Fig. 2). The only other radioactive peak detected was pyridine. After 48 h, accumulation of pigment in the medium was obvious and a major absorbance peak corresponding to riboflavin was observed at 18 min. Radioactivity was not detected in that peak, indicating that riboflavin was

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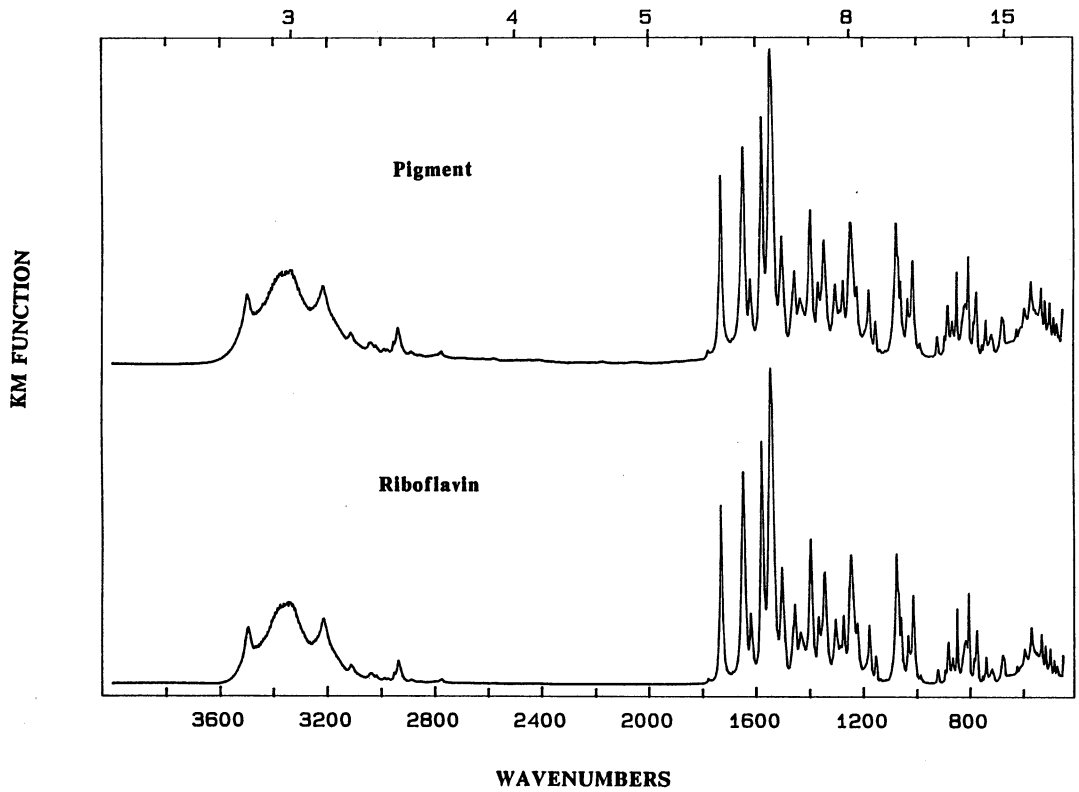

FIG. 1. Fourier transform infrared diffuse-reflectance spectra of isolated pigment and authentic riboflavin.

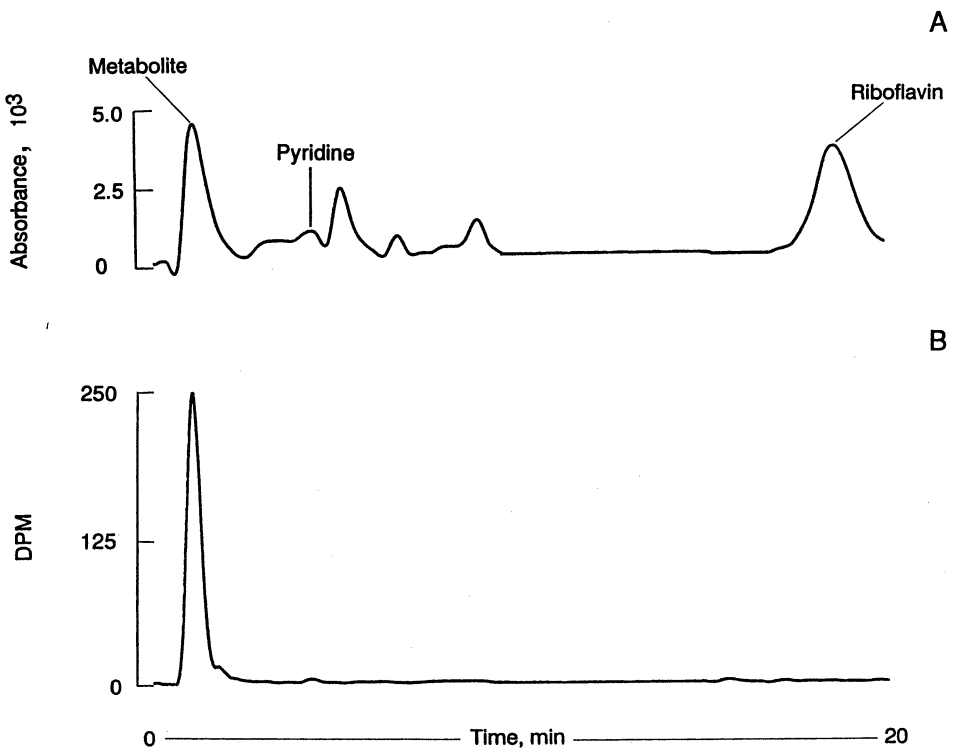

FIG. 2. HPLC chromatograms (A_{254} [A] and radioactivity [B]) of culture supernatant after 48 h of incubation.

not extensively labeled (Fig. 2). It should be noted that label would be expected to appear in riboflavin only if it was directly synthesized from pyridine since the labeled positions (carbons 2 and 6) are lost as formate or CO_2 during degradation of pyridine (6). Riboflavin might have been synthesized from the remaining three contiguous unlabeled pyridine carbons. In bacteria, riboflavin is synthesized from GTP, which is in turn synthesized from small molecules with no more than two contiguous carbons. Regardless of origin, riboflavin is probably not an intermediate in pyridine metabolism.

Riboflavin overproduction has been reported for fungi exposed to hydrocarbons (2, 4), and for a bacterium grown on 2-picoline (5). Other pigments, usually azaquinones or polymerization products, have been observed during microbial degradation of N heterocycles (6). It was not obvious what role, if any, riboflavin served in pyridine degradation by *M. luteus*. Pyridine may affect the ability of the organism to regulate riboflavin biosynthesis. Since riboflavin biosynthesis is thought to be controlled by coordinate repression in bacteria (1), interference with the regulatory mechanism would likely result in overproduction.

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